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Review

The role of natural environments in the evolution of resistance traits in pathogenic bacteria

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Antibiotics are among the most valuable compounds used for fighting human diseases. Unfortunately, pathogenic bacteria have evolved towards resistance. One important and frequently forgotten aspect of antibiotics and their resistance genes is that they evolved in non-clinical (natural) environments before the use of antibiotics by humans. Given that the biosphere is mainly formed by micro-organisms, learning the functional role of antibiotics and their resistance elements in nature has relevant implications both for human health and from an ecological perspective. Recent works have suggested that some antibiotics may serve for signalling purposes at the low concentrations probably found in natural ecosystems, whereas some antibiotic resistance genes were originally selected in their hosts for metabolic purposes or for signal trafficking. However, the high concentrations of antibiotics released in specific habitats (for instance, clinical settings) as a consequence of human activity can shift those functional roles. The pollution of natural ecosystems by antibiotics and resistance genes might have consequences for the evolution of the microbiosphere. Whereas antibiotics produce transient and usually local challenges in microbial communities, antibiotic resistance genes present in gene-transfer units can spread in nature with consequences for human health and the evolution of environmental microbiota that are largely ignored.

Keywords: antibiotic resistance; environmental micro-organisms; antibiotic pollution; bacterial evolution; microbial ecology; infectious diseases

1. INTRODUCTION

Since their introduction for human therapy 60 years ago, antibiotics have shown to be a remarkable success and constitute one of the most relevant medical inventions for reducing human morbidity and mortality. Unfortunately, the intensive use and misuse of antibiotics have resulted in antibiotic resistance among several human pathogens, reducing the possibilities for infections' treatment and jeopardizing medical procedures, such as organ transplantations or implants of prostheses, where infective complications are common and antibiotic therapy is needed to prevent or treat those infections (WHO 2000).

There are two main mechanisms involved in the development of antibiotic resistance, namely mutation (Martinez & Baquero 2000) and acquisition of resistance genes (Davies 1994) by horizontal gene transfer (HGT). Given that human pathogens were susceptible to antibiotics before the use of these drugs for the treatment of infections, the origin of antibiotic resistance determinants acquired by HGT must necessarily lay in the non-pathogenic microbiosphere. In some instances, human commensals can provide antibiotic resistance to pathogens (Sibold *et al.* 1994). However, in most cases, the antibiotic resistance genes have originated in the environmental microbiota (Davies 1994, 1997). The term antibiotic was originally coined to name those compounds produced by micro-organisms and capable of inhibiting bacterial growth (Waksman & Woodruff 1940), although any type of drug (natural or synthetic) used for treating bacterial infections is frequently termed as an antibiotic nowadays. Since several antibiotics are produced by environmental bacteria, it is conceivable that antibiotic-producing organisms could be the origin of HGT-acquired antibiotic resistance genes, because these micro-organisms must have systems to avoid the activity of the antimicrobials they produce (Benveniste & Davies 1973). Although this has been formally demonstrated in few occasions (Pang *et al.* 1994), we will see later that some resistance genes may have other functional roles in their original organisms besides antibiotic resistance (Martinez *et al.* 2009*a*).

Independent of their functional role in non-clinical (natural) environments, what is clear is that antibiotic resistance genes originated in environmental bacteria, so that changes in natural ecosystems may impact upon antibiotic resistance and consequently human health. Among those changes, the release of antibiotics together with human-linked microbiota eventually containing antibiotic resistance genes can be particularly important for the future evolution of antibiotic resistance in pathogenic bacteria (Baquero *et al.* 2008). However, this is just one side of the coin. Some micro-organisms are capable of producing infections only in people who are immunosuppressed (for instance, those with AIDS, under chemotherapy or after transplantation), debilitated

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or with a basal disease. These organisms, which do not usually infect healthy people, are considered as opportunistic pathogens (Quinn 1998; Gaynes & Edwards 2005). In some instances, human commensal bacteria produce opportunistic infections. Nevertheless, some environmental bacteria are also prominent opportunistic pathogens. One of the most problematic characteristics of opportunistic pathogens with an environmental origin is that they usually display low susceptibility to antibiotics (Hancock 1998; Quinn 1998; Hancock & Speert 2000; Ferrara 2006; Breidenstein *et al.* 2008; Martinez *et al.* 2009*b*). This is a common characteristic of all members of a given bacterial species. For instance, all the strains of *Pseudomonas aeruginosa* contain the same chromosomally encoded multidrug (MDR) efflux pumps (Alonso *et al.* 1999) and *ampC* beta-lactamase genes that contribute to the intrinsic phenotype of resistance displayed by this bacterial species (Bonomo & Szabo 2006). This indicates that those resistance determinants are ancient elements present in the chromosomes of all members of each bacterial species before its subspecific diversification some hundreds of thousands of years ago. This is consistent with the concept that the phenotype of intrinsic resistance to antibiotics was acquired in natural environments, outside clinical wards, long ago and is not the result of recent antibiotic usage for human therapy or farming. As stated for efflux pumps, these elements have predated the antibiotic era and their natural role is unlikely to be related to antibiotic use. For instance, synthetic antibiotics such as quinolones are unlikely to have selected for efflux pumps' evolution (Piddock 2006*a*).

All these features justify the concept that natural environments are important for the evolution of antibiotic resistance in bacterial pathogens. To understand which mechanisms have been and are currently being involved in this evolution, we will review the ecological roles of antibiotic and resistance genes in natural ecosystems and consequently which are the forces that shaped the evolution of resistance determinants before human use of antibiotics. We will review as well how bacterial pathogens acquire resistance from environmental bacterial and finally we will discuss the consequences of the release of antibiotics and resistance genes in natural ecosystems for the current and future evolution of resistance in bacterial pathogens.

2. FUNCTIONS OF ANTIBIOTICS AND ANTIBIOTIC RESISTANCE GENES IN NATURAL ECOSYSTEMS

Within this review, we will dub as 'natural ecosystems' or 'environmental bacteria' those that do not have a human-associated (commensal or infective) evolutionary linkage. Although natural ecosystems can suffer antibiotic pollution as the consequence of human activity, environmental bacteria are not globally under the strong antibiotic selective pressure suffered by human pathogens, which are challenged with antibiotics during therapy. One important feature to properly analyse antibiotic resistance in natural ecosystems is to understand the function of antibiotics and their resistance elements in those habitats. Since several of the antibiotics used for treating infections are synthesized by soil micro-organisms, it has been assumed that the function of these compounds in nature should be to inhibit the growth of the microbial competitors of the antibiotic producers (Waksman & Woodruff 1940). Conversely, it has been proposed that antibiotic resistance determinants have specifically evolved to avoid the activity of antibiotics, being the antibiotic producers a major source of resistance genes (Benveniste & Davies 1973).

Whereas in some occasions, these weapon/shield roles are reasonable explanations for the functions in nature of, respectively, antibiotics and resistance determinants (de Lorenzo *et al.* 1984; Pang *et al.* 1994), some other roles have been proposed for them. It has been discussed that some antibiotics may serve for signalling purposes, at the low concentrations probably encountered in most natural environments (Davies 2006; Yim *et al.* 2006*b*, 2007; Fajardo & Martinez 2008). This proposal is based on experimental work showing that low concentrations of antibiotics trigger specific transcriptional changes, which are independent of the general stress response microbial networks (Goh *et al.* 2002; Tsui *et al.* 2004; Linares *et al.* 2006; Yim *et al.* 2006*a*). Inasmuch, some compounds previously characterized as *bona fide* signal molecules also have antimicrobial activity (Ji *et al.* 1997; Deziel *et al.* 2004; Kaufmann *et al.* 2005), further supporting the dual role of these compounds.

Altogether, these studies indicate that the primary function of some antibiotics might be intercellular signalling in natural ecosystems, being inhibitors of bacterial growth only at the high concentrations used for therapy.

Concerning antibiotic resistance genes, some of them, currently present in pathogenic bacteria, are involved in antibiotic detoxification in the producer organisms (Pang *et al.* 1994) or in the resistance to toxic compounds produced by plants or by their associated microbiota (Bais *et al.* 2005, 2006), a feature that fits well with their role as shields. However, avoiding antibiotic activity is not always the primary role of some well-known antibiotic resistance determinants in natural ecosystems. For instance, it has been suggested that plasmid-encoded beta-lactamases, which are very proficient antibiotic resistance determinants acquired by pathogenic bacteria through HGT, might originally have been penicillin-binding proteins involved in the synthesis of peptidoglycan, and their activity against beta-lactams is a side effect of their original function (Kelly *et al.* 1986; Massova & Mobashery 1998; Meroueh *et al.* 2003). Similarly, the chromosomal 2′-*N*-acetyltransferase of *Providencia stuartii*, an enzyme involved in the modification of the bacterial peptidoglycan, can inactivate gentamycin and is considered as an antibiotic resistance determinant, although its original primary function is a different one (Macinga & Rather 1999). These examples illustrate the concept that a determinant which contributes to the resistance of human pathogens to antibiotics can be involved in central metabolic processes of environmental bacteria in their natural habitats. As shown here, a metabolic enzyme with a substrate presenting structural similarities to a given antibiotic might modify this antibiotic, thus serving as an antibiotic resistance gene in environments with a high antibiotic load, such as clinical settings.

Other mechanisms that do not involve antibiotic degradation were probably selected as well in nature to play a primary functional role different from antibiotic resistance. This is exemplified by the resistance to quinolones, a synthetic family of antibiotics introduced

for therapy in the 1960s. In spite of their synthetic origin, quinolones are a favourite substrate of MDR efflux pumps, and environmental bacteria, isolated before the invention of quinolones, can efflux these drugs (Alonso *et al.* 1999), indicating that resistance is not the primary function of those determinants. A similar situation might happen with Qnr, the first plasmid-encoded quinolone resistance determinant (Martinez-Martinez *et al.* 1998). It has been shown that *qnr* genes are chromosomally encoded in waterborne bacteria (Poirel *et al.* 2005; Sanchez *et al.* 2008). Furthermore, the high conservation in the synteny of the regions surrounding these genes together with the lack of elements associated to transposition or insertion events in their vicinity (Sanchez *et al.* 2008) supports the notion that these determinants originated in waterborne bacteria (Poirel *et al.* 2005), in habitats where the presence of quinolones is not expected.

As in the case of antibiotics, which can have a primary role different from killing competitors, we can then conclude that the primary functions in natural ecosystems of some resistance genes (e.g. metabolic enzymes or efflux pumps involved in signal trafficking) are not to avoid the activity of antibiotics.

3. INTRINSIC RESISTANCE

Antibiotic resistance has been mainly considered as an evolutionary process driven by antibiotics' selective pressure: bacteria are challenged with antibiotics and develop resistance as the consequence of mutation (Martinez & Baquero 2000) or HGT (Davies 1994). In fact, given the time required for the evolution of Metazoans (Navas *et al.* 2007), emergence and spread of resistance is considered as one of the few evolutionary processes that can be studied in real time (Salmond & Welch 2008). In spite of these considerations, the recent use (in evolutionary terms) of antibiotics for human therapy and farming is not the unique force driving evolution towards antibiotic resistance of human pathogens. Some bacterial species, presenting intrinsic low susceptibilities to antibiotics, have an environmental origin in habitats where there is not a high antibiotic load. Furthermore, those responsible for infections in hospitals, such as *P. aeruginosa*, *Acinetobacter baumannii* or *Stenotrophomonas maltophilia* (Quinn 1998), are not themselves producers of classical antibiotics and thus do not need to carry resistance genes in their genomes. A *naive* explanation to justify this resistance should be based on the fact that the target organisms used in the screening of antibiotics were the classical human pathogens, so that it would not be rare that environmental bacteria are able to resist those compounds. However, several antibiotics are broad spectrum, and their targets are present in environmental bacteria as well as in human pathogens. An alternative hypothesis emerges if we consider antibiotics as regular metabolites that have other functions besides killing competitors as discussed above.

Free-living bacteria such as those aforementioned have large genomes that allow them to colonize different environments (Cases *et al.* 2003) and on occasion possess a large number of biodegradative enzymes, which may cooperate in the degradation and use of antibiotics as food resource (Dantas *et al.* 2008). Besides, they form complex communities that require intercellular communication, and the mechanism for signal trafficking can be involved in resistance if the mechanisms for transporting the signal outside bacteria are co-opted by a given antibiotic. This is the case of MDR efflux pumps (Martinez *et al.* 2009*b*), a family of transporters that are present in all organisms (Saier & Paulsen 2001; Piddock 2006*a*; Lubelski *et al.* 2007), with the same chromosomally encoded MDR pumps being present in all strains of a given species (Alonso *et al.* 1999; Alonso & Martinez 2001; Sanchez *et al.* 2004). It has been shown that besides intrinsic antibiotic resistance and resistance against other toxic compounds (Silver & Phung 1996; Hernandez *et al.* 1998; Ramos *et al.* 2002; Sanchez *et al.* 2005), MDR pumps can contribute to virulence (Piddock 2006*b*) and are capable of efflux of intercellular signal compounds (Evans *et al.* 1998; Kohler *et al.* 2001).

Finally, intrinsically resistant environmental bacteria can colonize the rhizosphere, a habitat that might contain toxic compounds produced by either the plants or the associated microbiota (Matilla *et al.* 2007). The large metabolic versatility of free-living pathogens, which allows the colonization of such diverse habitats (Cases *et al.* 2003), probably allows them also to resist the toxicity of several compounds, including antibiotics (Gonzalez-Pasayo & Martinez-Romero 2000; Matilla *et al.* 2007; Martinez *et al.* 2009*b*). The complexity of the mechanisms involved in intrinsic resistance is further exemplified by studies showing that a large number of genes, belonging to different functional categories, contribute to intrinsic resistance to antibiotics (Breidenstein *et al.* 2008; Fajardo *et al.* 2008; Tamae *et al.* 2008).

All these considerations justify the conclusion that the phenotype of intrinsic resistance displayed by some opportunistic pathogens with an environmental origin has been acquired, during the evolution of these micro-organisms in their natural habitats, long before human discovery of antibiotics.

4. ACQUIRED RESISTANCE, AN EVOLUTIONARY PERSPECTIVE

In contrast to intrinsic resistance, which has been developed by bacterial populations before human use of antimicrobials, acquired antibiotic resistance is a recent event in the evolution of human pathogens in which the main selective force has been the human use of antibiotics (Levy 1998; Levy & Marshall 2004; Alekshun & Levy 2007; Salmond & Welch 2008). This view was earlier supported by the studies of Naomi Datta showing that the families of plasmids present in enterobacteria before and after the use of antibiotics are the same, and the only difference is that those plasmids have acquired resistance genes after antibiotics were introduced for therapy (Datta & Hughes 1983). As stated above, antibiotic resistance can be acquired either by mutation or by HGT. Whereas mutation-driven resistance can happen during antibiotic treatment, HGT-acquired resistance requires a donor of the resistance genes, so that the first transfer event requires contact between an environmental micro-organism (or its DNA in the case of transformation) and a human-associated bacteria. Although we will mainly refer to the acquisition of resistance by bacterial pathogens, it is worth remembering that commensal bacteria can serve as vectors for the transmission of resistance genes between

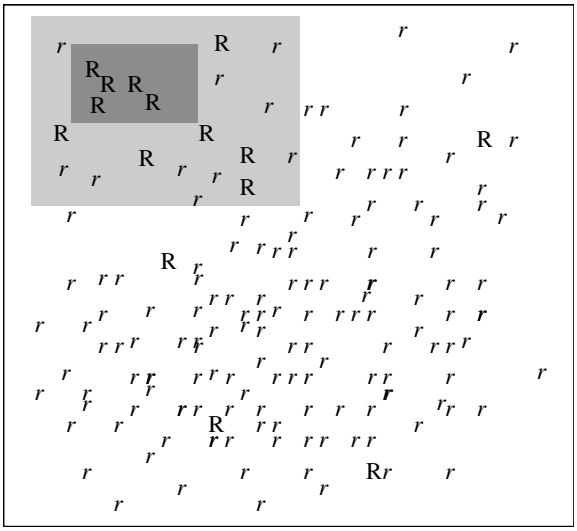

Figure 1. Ecological landscapes in antibiotic resistance. The determinants that can contribute to antibiotic resistance in pathogenic bacteria are distributed in all types of environments. However, neither their functional role nor the antibiotic selective pressure is the same in all of these environments. In natural (non-clinical) ecosystems (white square), antibiotic concentrations are usually low. Some determinants (R) can serve for antibiotics' detoxification in microbial producers or to avoid the inhibitory activities of antibiotics at places in which the concentrations of these drugs are locally high. However, some others (*r*), which can contribute to resistance in habitats with a high antibiotic load, are involved in metabolic or signalling processing in their original hosts. Opposite to this situation, the main functional role of these elements in habitats with a high antibiotic selective pressure such as clinical settings (dark grey square) will be resistance. Non-clinical environments receiving wastes containing antibiotics and human-linked (or animal-linked in the case of farms) bacteria (grey square) will be enriched in genetic platforms containing resistance genes already selected in bacterial pathogens, whose function is just resistance (see text for more details).

environmental and pathogenic micro-organisms. This situation implies the existence of three different landscapes important in the evolution of resistance of human pathogens (figure 1).

The first level is the whole microbiosphere. Environmental bacteria contain a large number of genes capable of conferring antibiotic resistance to human pathogens upon their transfer through HGT (D'Acosta *et al.* 2006; Martinez *et al.* 2007; Wright 2007). As discussed above, some of these determinants have been selected either for detoxification purposes in antibiotic producers, or to avoid the activity of plants' or micro-organisms' synthesized antimicrobials, whereas others have evolved to play functions different from antibiotic resistance. In any case, strong selective pressure by human-produced antibiotics did not have any relevance in the primary evolution of these determinants in natural ecosystems. Nevertheless, the release of high amounts of antibiotics and their resistance genes as a consequence of human activities in natural ecosystems has changed this situation in the last decades (see below).

The second level consists of those habitats with frequent contact of human-associated bacteria with the environmental microbiota, for instance wastewaters. Since HGT requires the two partners to be present in the same place, these allocations will be hotspots for the first step in the acquisition of antibiotic resistance genes by pathogenic bacteria: their transfer from the environmental bacteria to the human-associated ones. Furthermore, human-associated wastewaters frequently contain antibiotics and resistance genes, which increase the probabilities for DNA exchange and for the selection of resistance (Baquero *et al.* 2008).

The third level is the treated patient itself (or the animal in the case of antibiotics used for farming purposes). DNA transfer from commensals to pathogenic bacteria (for instance, in the development of resistance to beta-lactams by Gram-positive bacteria (Spratt *et al.* 1992; Sibold *et al.* 1994; Reichmann *et al.* 1997)) and between pathogenic bacteria in the case of polymicrobial infections has been described (Martinez-Suarez *et al.* 1987). However, the most important mechanism for developing resistance during infections is probably mutation (Macia *et al.* 2005). Actually, mutants with higher mutation rates than those of the wild-type strains are selected in clinical habitats (LeClerc *et al.* 1996; Oliver *et al.* 2000; Baquero *et al.* 2004) probably by a second-order selective process, which favour their evolution towards resistance in those environments with a strong antibiotic selective pressure (Macia *et al.* 2005). Under treatment, it can be thus predicted that acquisition of resistance mutations and mutation-driven diversification of genes, already acquired by HGT, will be the most relevant evolutionary pathways of bacterial pathogens.

Since the antibiotic selective pressure is different in these three environments, we can conclude that the relevance of the function of these elements as 'resistance determinants' is different as well. In the first one (natural ecosystems), antibiotic concentrations are not globally high, although they might be locally relevant, and the main function of those determinants would not necessary be resistance. Contrarily, in the treated host, with a high antibiotic load, HGT-acquired resistance determinants do not have either the genetic regulatory network nor the biochemical partners (substrates, other enzymes from the same metabolic pathway) they have in their original host, and their unique function will be just resistance (Martinez 2008), in an example of co-optive evolution, or exaptation (Gould & Lloyd 1999).

The evolutionary trajectory of an antibiotic resistance gene from an environmental micro-organism to become a *bona fide* resistance determinant in pathogenic bacteria presents two bottlenecks. The first one consists of its transfer and establishment in the human-associated microbiota. The use of culture-based (D'Acosta *et al.* 2006; Wright 2007) and metagenomics (Handelsman 2004; Mori *et al.* 2008; Allen *et al.* 2009*a*,*b*) methods to analyse environmental microbiota has demonstrated that the number of potential resistance determinants in environmental bacteria is huge. However, very few of them are currently present in human pathogens (Martinez *et al.* 2007). Four elements contribute to this situation. First, only those genes that can coexist with human pathogens are suitable to contribute to resistance. For instance, it is very unlikely that resistance genes found in organisms from the deep terrestrial subsurface (Brown & Balkwill 2008), or present in the deep Greenland ice core (Miteva *et al.* 2004), might contribute to resistance of bacterial pathogens. Second, only those genes recruited by

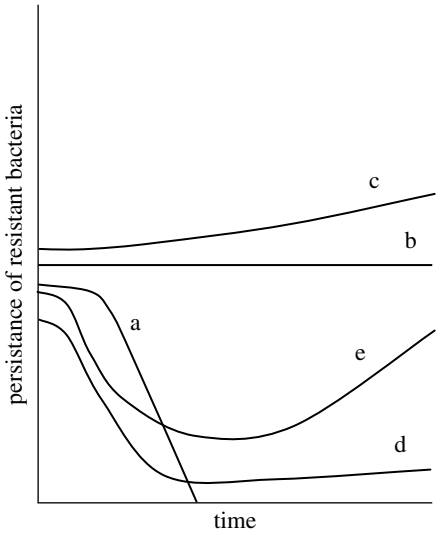

Figure 2. Effect of antibiotic resistance on bacterial fitness. The acquisition of a phenotype of resistance is frequently linked to a fitness cost, which results in resistant populations being outcompeted by susceptible ones in the absence of antibiotics (a). However, some antibiotic resistance mutations have no cost (b) and resistant bacteria are maintained, or are even beneficial by increasing bacterial fitness (c), in which case the resistant bacteria prevail over susceptible ones even in the absence of antibiotics. Furthermore, resistant bacteria can acquire compensatory mutations that increase their fitness to the level of the susceptible population (d), so that these compensated mutants are maintained, or eventually increase their fitness (e) and the compensated mutants outcompete the susceptible bacteria even in the absence of antibiotics.

gene-transfer systems compatible with human pathogens will be transferred. The transfer of these systems is limited by barriers that include their host range (e.g. the hosts in which the plasmids can replicate), the exclusion between different plasmids and the DNA restriction/modification systems, among others (see Thomas & Nielsen (2005) for a review in depth of those mechanisms). Direct transformation can be also important for naturally competent pathogens. In this case, the maintenance of the transferred DNA requires its integration in the recipient chromosome. The probability of this recombination event varies as a function of the organism (e.g. 0.1% of the internalized fragments are integrated in *Acinetobacter baylyi*, whereas more than 25% of them are successfully recombined in *Bacillus subtilis* and *Streptococcus pneumoniae* (Thomas & Nielsen 2005)) and the homology between the incoming DNA and the regions of the chromosome of the recipient cell involved in recombination (Lovett *et al.* 2002; Thomas & Nielsen 2005). Mutator strains, which display a high mutation rate, are also highly recombinogenic (Matic *et al.* 2000). Third, those elements that produce strong fitness costs in their hosts (figure 2) will be counterselected (Andersson & Levin 1999; Morosini *et al.* 2000), unless compensatory mutations are easily selectable (Bjorkman *et al.* 2000; Paulander *et al.* 2007; Lofmark *et al.* 2008). Fourth for those elements that fulfil the three former criteria a founder effect is expected (Martinez *et al.* 2009*a*), in such a way that the first resistance determinant acquired by HGT will spread and the probabilities for the acquisition of a new one will be low, unless antibiotic selective pressure changes.

The second bottleneck consists of the strong selective pressure (mainly during antibiotic therapy) suffered by pathogenic bacteria. This selective pressure can lead to the diversification of the HGT-acquired genes. TEM beta-lactamases are a good example of this process. TEM-1, the first member of this family of enzymes, inactivates the first class of beta-lactams and has been prevalent for decades in resistant enteric Gram-negative bacilli (Roy *et al.* 1983). After the introduction of beta-lactamase inhibitors and new generations of beta-lactams, a strong diversification in TEM-1 derived enzymes has occurred (Paterson & Bonomo 2005) to the point that the number of 'mutant' TEM enzymes are now above one hundred. A similar situation is currently occurring with other beta-lactamases such as those belonging to the CTX-M family. The finding of two CTX-M derivatives surrounded by identical sequences in the same IncN plasmids indicates that the evolution of these enzymes has occurred after their integration in those plasmids (Novais *et al.* 2007). These examples support the conclusion that those new (evolved) resistance genes were not present before in nature and their emergence is a direct consequence of the evolutionary process triggered by human use of antibiotics. Whether or not their reintroduction in natural ecosystems may challenge the environmental microbiota is a topic that has not been properly addressed.

5. CHANGES IN NATURAL ECOSYSTEMS AND FUTURE EVOLUTION OF ANTIBIOTIC RESISTANCE

Since antibiotic resistance genes originated in environmental bacteria, an important topic to address would be to state whether changes in the natural ecosystems, mainly as the consequences of human activities, can challenge the environmental microbiota in such a way that these modifications alter the resistance of human pathogens (Alonso *et al.* 2001).

Antibiotics are nowadays used, not just for human therapy, but also for farming purposes. This includes the treatment of infections, and their use for promoting faster growth of livestock (Ferber 2003). Although the usage of antibiotics for this last purpose has been banned in some countries, the release of antibiotics that are not used for preventing or treating human infections is still very high. Together with the antibiotics used in clinics, this has meant the contamination with antibiotics at farms, rivers that receive wastewaters and lands receiving antibiotic-contaminated manure occurs frequently (Cabello 2006). This can accelerate evolution towards resistance and increases the risks for its transfer to human pathogens, which can be present as well in these ecosystems (Baquero *et al.* 2008). For instance, the presence of plasmid-encoded *qnr* genes in environmental *Aeromonas* spp. has been recently reported (Cattoir *et al.* 2008). Since these determinants are chromosomally encoded in waterborne bacteria, their integration in a gene-transfer element, may be as the consequence of the use of quinolones in aquaculture and could be a first step for its transfer to human pathogens. Notably, the same plasmid containing the same *qnr* gene has been found in geographically distant allocations (Picao *et al.* 2008), indicating that once antibiotic resistance genes are integrated in gene-transfer elements, they then have a better chance of dissemination and to eventually remain in bacterial populations.

Another type of contamination that can be relevant for the evolution of resistance in bacterial pathogens is constituted by the antibiotic resistance genes themselves. The finding of resistant organisms worldwide should not be taken as a surprise, given the environmental origin of resistance genes (Davies 1994). What is problematic however is the finding of resistance determinants, already well established in bacterial pathogens, in environments without a history of antibiotic contamination (Pallecchi *et al.* 2008). For instance, remote human populations without known antibiotic exposure carry antibiotic resistant bacteria (Grenet *et al.* 2004; Bartoloni *et al.* 2009) and resistant bacteria are also established in wild animal populations, despite not receiving any antibiotic (Gilliver *et al.* 1999; Livermore *et al.* 2001).

The presence of the same antibiotic resistance genes, associated with the same genetic platforms (integrons, transposons and plasmids) in both bacterial pathogens and pristine environments with non-detectable concentrations of antibiotics, implies the existence of a worldwide-distributed antibiotic resistance background. These resistance determinants are already present in the environment in genetic platforms compatible with bacterial pathogens and thus ready to be transferred by HGT under antibiotic selection.

This suggests that some antibiotic resistance genes are difficult to eliminate even in the absence of antibiotic selective pressure, and thus remain in the environment modifying the genetic repertoire and eventually the population dynamics of environmental micro-organisms.

There are different reasons for explaining the maintenance of resistance-transfer units in the absence of selective pressure.

Some resistance mechanisms have no cost for the bacteria, or even render a higher fitness in specific environments (Alonso *et al.* 2004; Luo *et al.* 2005; Balsalobre & de la Campa 2008), in such a way that resistant micro-organisms are not outcompeted by their wild-type counterparts (figure 2). Even for relevant fitness costs, compensatory mutations reducing bacterial burden might be selected (Bjorkman *et al.* 2000; Paulander *et al.* 2007; Lofmark *et al.* 2008).

Several plasmids encode toxin–antitoxin systems (Jaffe *et al.* 1985; Gerdes *et al.* 1986; Hiraga *et al.* 1986; Hayes 2003). If those plasmids contain antibiotic resistance genes (Moritz & Hergenrother 2007; Perichon *et al.* 2008; Sletvold *et al.* 2008), the probability for the maintenance of resistance might be high (figure 3).

Antibiotic resistance genes can be clustered in the same unit (for instance an integron (Stokes & Hall 1989; Mazel 2006)) with other elements that can confer an ecological advantage (figure 3). This includes resistance to toxic compounds (e.g. antibiotics, heavy metals, biocides) and other elements, such as siderophores, microcins or toxins, which can favour the survival of bacteria in a given habitat (Delgado-Iribarren *et al.* 1987; Martinez & Perez-Diaz 1990; Herrero *et al.* 2008). Under these circumstances, resistance can be co-selected by its associated trait in the absence of antibiotics. Since heavy metal resistance and biocide resistance genes can be associated with antibiotic resistance, this implies that heavy metal contamination, as well as the usage of biocides, might select for antibiotic resistance in natural environments (Novick & Roth 1968; Foster 1983; Morozzi *et al.* 1986; Belliveau *et al.* 1991; Summers *et al.* 1993; Dhakephalkar & Chopade 1994; Hernandez *et al.* 1998; McArthur & Tuckfield 2000; Gaze *et al.* 2005; Sanchez *et al.* 2005; Stepanauskas *et al.* 2006).

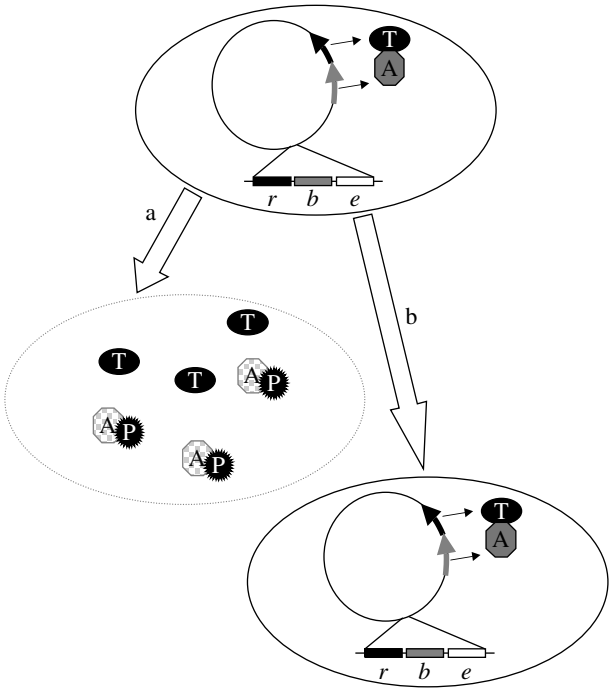

Figure 3. Maintenance of antibiotic resistance platforms in the absence of antibiotic selective pressure. Antibiotic resistance genes are acquired by genetic platforms that can disseminate among bacterial populations. The Figure represents a bacterium carrying one plasmid. The plasmid can contain different genes, which encode determinants with adaptive values for the recipient bacterium. This includes antibiotic resistance genes (*r*), biocide resistance or heavy metal resistance determinants (*b*) or ecologically rewarding elements (*e*) such as siderophores, microcins or toxins among others. Even in the absence of antibiotics, the presence of these determinants associated in the same plasmid can favour its selection and thus co-selection of antibiotic resistance. On the other hand, several plasmids express toxin (T)/antitoxin (A) systems that impede their loss. The antitoxin binds the toxin and thus impedes bacterial death. However, if the plasmid is lost (a), the production of those two proteins is stopped and the antitoxin is rapidly degraded by a protease (P), liberating the toxin and allowing bacterial killing. If the plasmid is maintained (b), the antitoxin remains to be continuously produced and the activity of the toxin is inhibited. If this type of plasmids contains resistance genes, their loss, even in the absence of antibiotic selective pressure, is unlikely.

Most works on the effects of human activity on the biosphere are based on the study of higher organisms. However, the majority of life is microbial, and the effects of environmental changes on the microbiosphere are largely ignored. We know that pollution by antibiotics and antibiotic resistance genes can alter the environmental microbiota. Nevertheless, we ignore whether part of these alterations might remain over the long term. Whereas antibiotics are degraded in nature, the genetic platforms containing resistance genes are auto-replicative elements that might be rather stable. Several antibiotics and resistance genes have an environmental origin in habitats where they have evolved without contact with humans for hundreds of thousands of years. On occasions, the selective force for the evolution of resistance genes in

natural environments has been the presence of a given toxic compound (not necessarily an antibiotic). However, at other times, the determinants that nowadays contribute to resistance of bacterial pathogens were selected for metabolic, structural or signal-trafficking purposes (see above) in habitats with low antibiotic selective pressure. The introduction by humans of antibiotics for therapy has changed this situation in habitats where the main selective force is now the presence of high concentrations of antibiotics. The enrichment, in populations of environmental bacteria, of genetic platforms containing antibiotic resistance genes as the consequence of this very recent (in evolutionary terms) human-linked antibiotic use, is accelerating the evolution and spread of resistance among microbial populations, including bacterial pathogens.

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